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VIRUS-CELL INTERACTIONS

Mosquito Defensins Enhance Japanese Encephalitis Virus Infection by Facilitating Virus Adsorption and Entry within the Mosquito

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ABSTRACT Japanese encephalitis virus (JEV) is a viral zoonosis that can cause viral encephalitis, death, and disability. Although the *Culex* mosquito is the primary vector of JEV, little is known about JEV transmission by this kind of mosquito. Here, we found that mosquito defensin facilitated the adsorption of JEV on target cells via the defensin/lipoprotein receptor-related protein 2 (LRP2) axis. Mosquito defensin bound the ED III domain of the viral envelope (E) protein and directly mediated efficient virus adsorption on the target cell surface; the receptor LRP2, which is expressed on the cell surface, affected defensin-dependent adsorption. As a result, mosquito defensin enhanced JEV infection in the salivary gland, increasing the possibility of viral transmission by mosquitoes. These findings demonstrate the novel role of mosquito defensin in JEV infection and the mechanisms through which the virus exploits mosquito defensin for infection and transmission.

IMPORTANCE In this study, we observed the complex roles of mosquito defensin in JEV infection; mosquito defensin exhibited a weak antiviral effect but strongly enhanced binding. In the latter, defensin directly binds the ED III domain of the viral E protein and promotes the adsorption of JEV to target cells by interacting with lipoprotein receptor-related protein 2 (LRP2), thus accelerating virus entry. Together, our results indicate that mosquito defensin plays an important role in facilitating JEV infection and potential transmission.

KEYWORDS Japanese encephalitis virus, mosquito defensin, adsorption, infection in mosquito, transmission potential

Japanese encephalitis virus (JEV), a member of the *Flaviviridae* family and *Flavivirus* genus, is prevalent in tropical and subtropical regions in Asia and the Pacific (1–3). JEV is mainly transmitted through mosquito bites (2, 4). Pigs serve as reservoir hosts for JEV, and humans, horses, and other animals are dead-end hosts (2, 5). Because the prevention and control of JEV rely on vaccines that offer a limited window of protection (6–8), JEV can easily cause death or permanent disability. A large number of people are at risk of JEV infection, with immunocompromised children and older individuals at particular risk (9, 10). The World Health Organization reported that more than 67,900 cases of JEV infection occur globally each year and that more than 10,000 of these cases are fatal. As the global temperature increases, the clinical incidence of Japanese encephalitis increases as well, owing to increases in the habitat range and activity of mosquitoes carrying JEV as the climate warms (2, 4, 9). Few studies have addressed the mechanism of JEV transmission by mosquito vectors (4). Thus, obtaining a detailed

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understanding of the interaction between JEV and mosquito vectors is essential to improve the control of JEV transmission.

The *Culex* mosquito is the principal vector of JEV (11, 12), which can spread throughout the mosquito body, including the salivary glands (13). When an infected mosquito bites a human or animal, the virus is transmitted to the human or animal skin tissue through the mosquito saliva. JEV also induces an immune response in the mosquito vector (14–16). For example, C-type lectin and a series of other proteins increase rapidly after infection (17, 18). C-type lectin plays an important role in infection by JEV and other flaviviruses in mosquitoes, but the role of defensin has not yet been clearly characterized.

Defensins are antimicrobial peptides consisting of 25 to 60 amino acids that are produced by the innate immune system (15, 19). Defensin is one of the crucial immune effectors in insects (20). Furthermore, the antiviral effects of defensins have been well described in mammalian cells. Human defensins have been reported to inhibit herpes simplex virus type 2 (retrocyclin-1, retrocyclin-2) (21), human immunodeficiency virus (human beta defensin-1, human beta defensin-2, human beta defensin-3) (22, 23), and other viruses. However, expression of human beta defensin-6 by an adenovirus vector enhanced parainfluenza virus type 3 replication (24). Normally, mammalian defensins can directly destroy virus particles by binding the surface of envelope (E) proteins. They can also interact with cell-surface receptors and influence cell signal transduction (19, 25). Although the mammalian and mosquito immune systems exhibit many differences, defensins are important effectors in the mosquito immune response. Therefore, the role of mosquito defensins during the process of JEV infection requires further study.

In this study, we observed the complex roles of mosquito defensin in JEV infection: mosquito defensin exhibited a weak antiviral effect but strongly enhanced binding. In the latter, defensin directly binds the ED III domain of the viral E protein and promotes the adsorption of JEV to target cells by interacting with lipoprotein receptor-related protein 2 (LRP2), thus accelerating virus entry. Together, our results indicate that mosquito defensin plays an important role in facilitating JEV infection and potential transmission.

RESULTS

JEV infection upregulated defensin expression *in vivo* and *in vitro*. Defensin is one of the major innate immunity effectors in mosquitoes. To study the role of defensin in JEV infection, we first assessed the JEV infection curve and the expression of defensin after JEV infection in *Culex pipiens pallens*, which is the natural vector of JEV. Five-day-old female mosquitoes after emergence were infected by administration of 1,000 50% mosquito infective dose (MID_{50}) via microinjector (18). The mosquitoes were collected 4, 7, and 10 days after injection, and JEV E mRNA levels in the whole body, salivary gland, and midgut were determined. JEV E mRNA levels were higher in the salivary gland than in the whole body or midgut (Fig. 1A). At day 10, the JEV E mRNA level increased dramatically, indicating that the virus reproduced rapidly during this period. Specifically, JEV E mRNA levels were 9.7- and 11.7-fold higher at day 10 than at day 7 in the whole body and midgut, respectively. A greater increase was observed in the salivary gland, reaching 14.9-fold at day 10 compared with day 7. JEV E mRNA levels after 4 days were much lower than those after 7 or 10 days, even though JEV E mRNA could be clearly detected by real-time PCR.

Because high virus levels in mosquitoes were observed 7 and 10 days after JEV infection, we then determined the defensin mRNA level in the whole body on days 7 and 10. At least two isoforms of defensin not found in the NCBI database were observed in *C. pipiens pallens*. Then, we identified and confirmed the sequences of defensin isoforms before their detection. Based on the results of PCR amplification (data not shown; data are available upon request), sequencing, and BLASTn search (https://blast.ncbi.nlm.nih.gov), we found two gene types of defensin, defensin A (submitted to NCBI with accession number MH756645) and a defensin with an incomplete sequence. The mature protein regions of these two defensins shared 99.5%

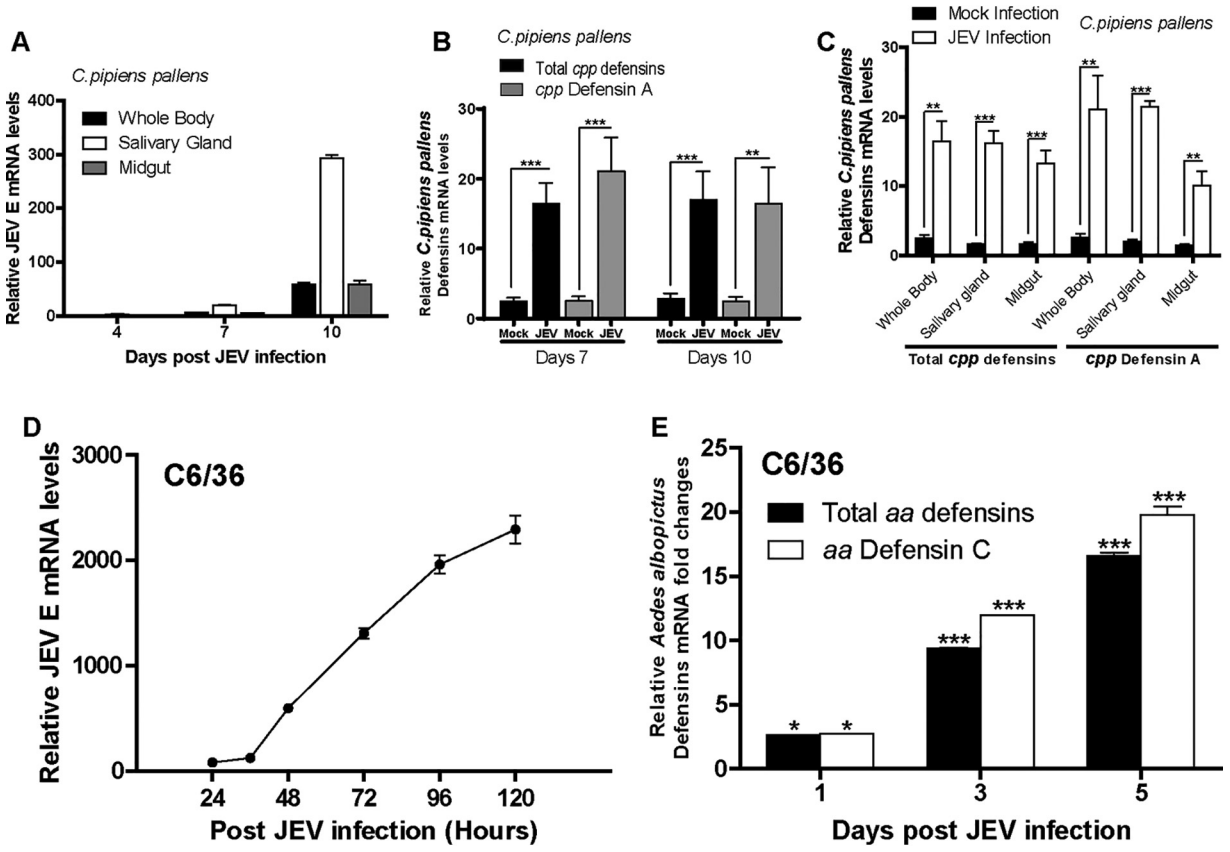

FIG 1 JEV infection upregulated defensin in mosquito vectors. (A) JEV infection curve in mosquitoes. JEV (10^3 MID_{50}) or PBS was inoculated into female mosquitoes by microinjection. Whole-body, salivary gland, and midgut samples were collected at 4, 7, and 10 days after JEV infection. JEV E expression was quantified by real-time PCR. (B) Expression levels of *C. pipiens pallens* (*cpp*) defensins in the whole body were determined. JEV (10^3 MID_{50}) or PBS was inoculated into female mosquitoes by microinjection. Total defensin and defensin A mRNA levels in the whole body at 7 and 10 days after JEV infection were quantified by real-time PCR. (C) Expression levels of *C. pipiens pallens* defensins in the midgut and salivary gland were determined. JEV (10^3 MID_{50}) or PBS was inoculated into female mosquitoes by microinjection. The midgut and salivary gland were separated at 7 days after infection. Total defensin and defensin A mRNA levels were quantified by real-time PCR. (D) One-step growth curve of JEV in C6/36 cells. C6/36 cells were infected with JEV at an MOI of 5 and collected at different time points. JEV E mRNA levels were quantified by real-time PCR. (E) Expression levels of *A. albopictus* (*aa*) defensins. C6/36 cells were infected with JEV at an MOI of 5 and collected at 1, 3, and 5 days after JEV infection. Total defensin and *A. albopictus* defensin C mRNA levels were quantified by real-time PCR. All experiments were performed in triplicate at least three times. Data are shown as the mean values ± standard deviations.

sequence similarity (data not shown). We designed primers specific to the *C. pipiens pallens* defensin A sequence (Table 1) for real-time PCR detection. However, no specific primers could be designed for the unnamed defensin because little of its specific sequence was obtained. Therefore, we quantified the mRNA copy number of total defensin and type A defensin to determine the proportion of type A defensin among all defensin in *C. pipiens pallens*. Type A defensin accounted for 53.36% of the total defensin. We then decided to use type A defensin as a representative defensin for further study. To analyze the correlation between defensins among organisms, we aligned the defensin protein sequences of mosquito vectors of flaviviruses. All sequence similarities between mosquito vectors were above 97.6%, implying that mosquito defensins carry out similar functions.

C. pipiens pallens defensin A mRNA levels on days 7 and 10 were significantly higher in the JEV infection group than in the control group, although the level was slightly lower at day 10 (Fig. 1B). The change in the level of total defensin followed a similar trend (Fig. 1B). Furthermore, we compared *C. pipiens pallens* defensin A and total defensin mRNA levels in the whole body, salivary gland, and midgut. Defensin A and total defensin mRNA levels were similar, and increased transcript abundance was observed in the salivary gland and whole body compared to the midgut (Fig. 1C).

TABLE 1 Primers and siRNA sequences

Gene	Sense primer	Antisense primer	Reference sequence GenBank accession no.
Real-time PCR			
C. pipiens pallens beta actin	GTGTGATGGTCGGTATGG	ACTCGCAACTCATTGTAGA	AY100005.1
C. pipiens pallens defensin A	ATGAACTCGCTTGGAACA	TTCGTCGAACAAAGTATTGG	MH756645
C. pipiens pallens universal	TCAGTCCTACGCCAATAC	TTGACGCCTAATCCACTC	Not submitted
C6/36 beta actin	CCTGGGTATGGAAGCCTGCGGTATC	GGCAATGATCTTGATCTTCATGGTGGATGG	[a]
C6/36 defensin C	ATGCGTTCTCTCAGTTTG	AGTTCATCAAACAGAGAGT	AAEL003832
C6/36 total defensins	TGATTTGTTTCCTGGCTAT	AGCTTGGTAGCATTCCTC	AAEL003841, AAEL003857
JEV	CCTCCGTCACCATGCCAGTCTTAG	TTCGCCATGGTCTTTTTCCTCTC	[a]
C6/36 LRP2	ACAAGCATACACATACACATACG	GCACAATCAGTCATCAATAGGT	XM_029854739.1
C. pipiens pallens LRP2	GCAGTTGTCTTATTCATA	TAACGATACTACACTTAGA	Not submitted[b]
RNA interference			
C. pipiens pallens total defensin siRNA-1	CCAAUACUUCGCCAAUACUTT		Not submitted
C. pipiens pallens total defensin siRNA-2	GCCAUCGUUUCCACUGGAATT		Not submitted
C. pipiens pallens defensin A siRNA-1	GCUUUGTGCCUGUUUGCCATT		MH756645
C. pipiens pallens defensin A siRNA-2	GCUUGGAACAGCUUGCUUGTT		MH756645
A. albopictus total defensin siRNA-1	GGAGGAAUGCUACCAAGCUTT		AAEL003841, AAEL003857
A. albopictus total defensin siRNA-2	GCGAUCUGCUGAGUGGAUUTT		AAEL003841, AAEL003857
A. albopictus defensin C siRNA-1	GGCAAACUCUCUGUUUGAUTT		AAEL003832
A. albopictus defensin C siRNA-2	GCAUUCCCUGAAGAGCUGUTT		AAEL003832
C6/36 LRP2 siRNA-1	CGAUUGUUUGGAUAAUAGUTT		XM_029854739.1
C6/36 LRP2 siRNA-2	CCAAUCAAUUUAAGUGUAATT		XM_029854739.1
C. pipiens pallens LRP2 siRNA-1	GUAACAAGACGCGCUUUUUTT		Not submitted[b]
C. pipiens pallens LRP2 siRNA-2	GCACGAACGTGGAGTTCAATT		Not submitted[b]

[a]The primers refer to a published article (18).
[b]*C. pipiens pallens* LRP2 primers and siRNA were designed according to a partial cloned mRNA sequence.

Significantly higher mRNA levels ($P < 0.005$) in the whole body, saliva, and midgut were observed in the JEV infection group than in the control group. This suggests that *C. pipiens pallens* defensin A expression is positively correlated with JEV infection in mosquito.

C6/36 cells, a cell line derived from *Aedes albopictus*, are commonly used in mosquito studies. Because the genomic information and background of C6/36 cells have been clearly shown, we used C6/36 cells as a cellular model in this study. Based on sequences from the NCBI database, we found three *A. albopictus* defensins with high homology to *C. pipiens pallens* defensins. We also quantified the mRNA copy number of total defensin and type C defensin to determine the proportion of type C defensin in *A. albopictus* cells (data not shown; data are available upon request). Type C defensin accounted for a high proportion (40.69%) of all defensins in C6/36 cells. Because of the high ratio of defensin C in *A. albopictus* and the high homology between *A. albopictus* defensin C and other mosquito defensins, we decided to use *A. albopictus* defensin C (GenBank accession number XP_019538951.2) to study the functions of defensin in JEV infection of C6/36 cells.

To confirm the upregulation of defensin in different mosquito vectors caused by JEV infection, we infected C6/36 cells with JEV *in vitro*. JEV E mRNA levels increased from 24 h to 120 h after JEV infection (Fig. 1D). We further analyzed the changes in total defensins and primary defensins (*A. albopictus* defensin C, data not shown) after JEV infection. *A. albopictus* defensin C mRNA levels were upregulated 2.75-, 11.9-, and 19.7-fold at days 1, 3, and 5, respectively, in cells infected with JEV compared with mock-infected cells (Fig. 1E). Additionally, total defensin levels were upregulated after JEV infection (Fig. 1E). Together, our results indicated that defensin levels were upregulated after infection both *in vivo* and *in vitro*.

Mosquito defensin shows species specificity in facilitating JEV infection. Mature defensins are extracellular proteins less than 60 amino acids in length. To confirm the function of mosquito defensin in JEV infection, we synthesized mature mosquito and human defensin peptides with a purity of ≥99% for further analysis (Table 2). *C. pipiens*

TABLE 2 Defensin sequences

Defensin	Peptide sequence[a]	NCBI accession no.
Culex pipiens pallens defensin A	ATCDLLSGLGVNDSACAAHCIARGNRGGYCNSKKVCVCRN	MH756645
scramble *Culex pipiens pallens* defensin A	CVNKRCHRDGSALCKCGYNSLGLDACRAAVGNCSGTNIVA	
Aedes albopictus defensin C	ATCDLLSGFGVGDSACAAHCIARRNRGGYCNAKKVCVCRN	XP_019538951.2
scramble *Aedes albopictus* defensin C	LSKCANIYDKCLSGRVVVCRDACCRGTFNCAAGRGAHANG	
Human defensin β2	VTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCK	NP_004933.1
Scramble human defensin β2	GPSQCFGCIYHVTCPCAKKVGTCRTKGKPRLICL	

[a]Mature defensins and scramble defensins were synthesized in a high purity (≥99%). FITC labeled defensin used in this study was synthesized by N-terminal labeling.

pallens defensin A and *A. albopictus* defensin C peptides were used in *C. pipiens pallens* and C6/36 cells, respectively. Scrambled defensin peptides were used as controls. Defensins (100 μM) and JEV (10 MID_{50}) were mixed before injection into mosquitoes. JEV E mRNA levels in the whole body at 7 and 10 days after JEV infection were quantified by real-time PCR. Unexpectedly, in the *in vivo* experiment, JEV E mRNA levels at 7 and 10 days after infection were increased by 2.95- and 6.13-fold, respectively, in the *C. pipiens pallens* defensin A-treated group compared with the control group (Fig. 2A). *A. albopictus* defensin C was also injected into *C. pipiens pallens*, and its effects were compared to those of *C. pipiens pallens* defensin A to analyze the functions of defensins from different species. The JEV level in the *A. albopictus* defensin C-treated group changed in the same way as that in the *C. pipiens pallens* defensin A-treated group (Fig. 2A). To avoid obtaining false results due to defensin peptide injection, we confirmed the mosquito defensin-mediated increase in JEV infection by *in vivo* RNA interference (RNAi). Small interfering RNA (siRNA) sequences targeting *C. pipiens pallens* defensins (targeting the homology domain of *C. pipiens pallens* defensin) or *C. pipiens pallens* defensin A (targeting a specific domain of *C. pipiens pallens* defensin A) were designed and used for *in vivo* RNAi. JEV was infected after a 3-day recovery period, and virus levels in the whole body at 3 days after JEV infection were quantified by real-time PCR. siRNA knockdown significantly decreased defensin mRNA in *C. pipiens pallens* (Fig. 2B). JEV E mRNA levels were decreased by more than 5-fold in the *C. pipiens pallens* defensin siRNA groups and more than 3-fold in the *C. pipiens pallens* defensin A siRNA groups compared to the scramble group (Fig. 2C).

To directly confirm this effect of mosquito defensin on JEV infection, we employed C6/36 cells, a mature mosquito cell line commonly used for *in vitro* assays. To confirm the effect of mosquito defensins in enhancing JEV infection, we transfected C6/36 cells with siRNAs targeting defensins (Fig. 2D). JEV was inoculated and detected after siRNA transfection. JEV E mRNA levels were decreased by 4.7- to 6-fold in the group in which total *A. albopictus* defensins were knocked down and decreased by 2.3- to 3.1-fold in the group in which *A. albopictus* defensin C was knocked down (Fig. 2E, i and ii). These results were consistent with the *in vivo* data. To directly observe the influence of mosquito defensin on JEV, we performed an immunofluorescence assay. Analysis by indirect immunofluorescence assay (IFA) also showed higher JEV E levels in the mosquito defensin-treated cells than in the control cells (Fig. 2F, upper) and lower JEV E levels in the mosquito defensin-knockdown cells than in the control cells (Fig. 2F, lower). These results imply that mosquito defensins facilitate JEV infection within mosquito species.

Its antimicrobial property is the main reported feature of defensin. To further determine the role of mosquito defensin in JEV infection among different species, the function of human defensin β2, which shows high antiviral activity, in JEV infection was compared to those of the other tested defensins (25, 26). First, we compared the effects of *A. albopictus* defensin C, *C. pipiens pallens* defensin A, and human defensin β2 on C6/36 cells. *A. albopictus* defensin C enhanced JEV infection of C6/36 cells, as indicated by both the JEV E mRNA levels (increased by 4.88-fold, $P < 0.001$) and 50% tissue culture infective dose ($TCID_{50}$) (1.3 titer, $P < 0.05$) (Fig. 2G, i and ii). Treatment with *C. pipiens pallens* defensin A also significantly enhanced JEV infection ($P < 0.05$). In contrast, human defensin β2 inhibited JEV replication on C6/36 cells ($P < 0.05$), dem-

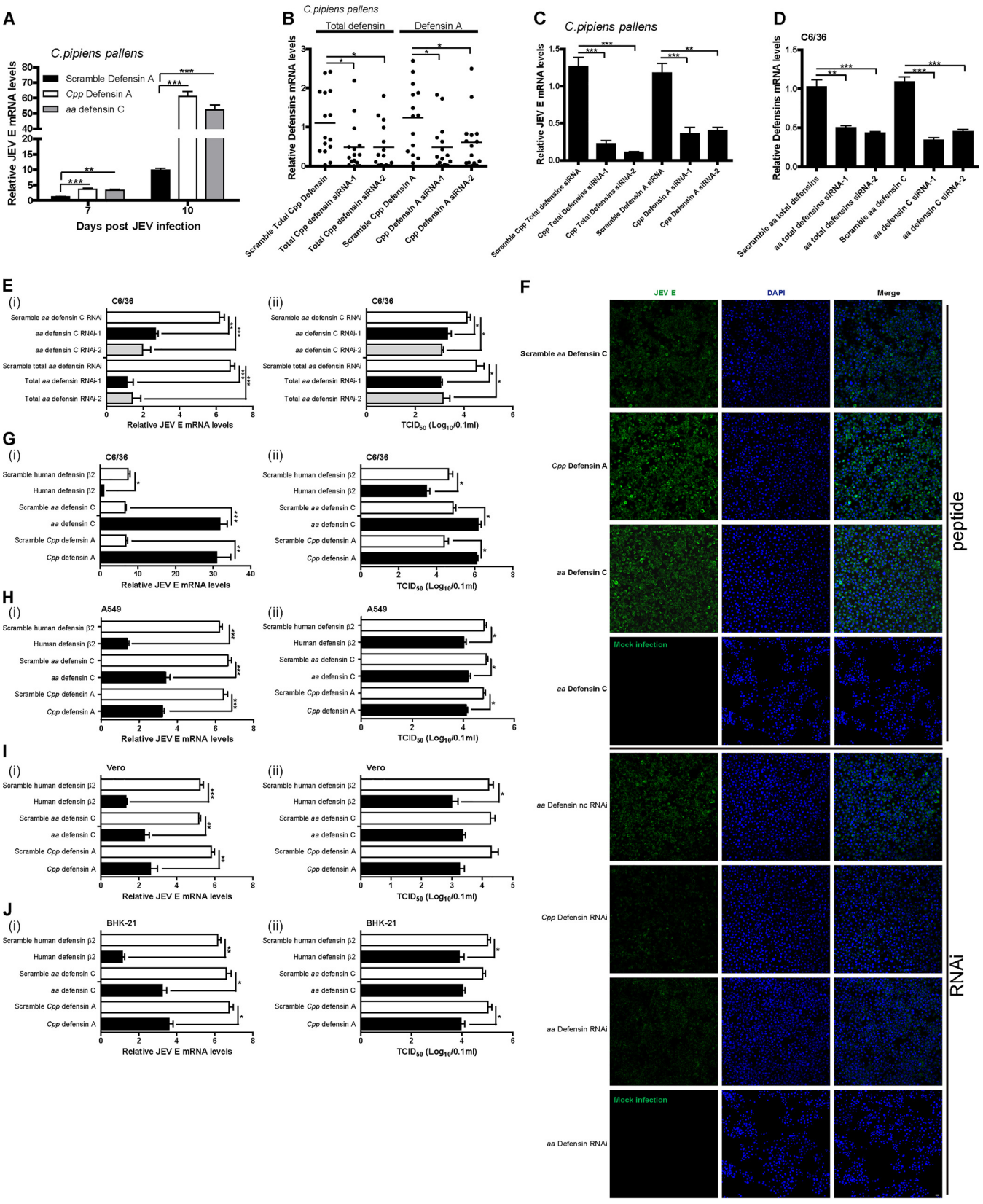

FIG 2 Mosquito defensin facilitated JEV infection in mosquito vectors. (A) Mosquito defensin facilitated JEV infection in *Culex* mosquitoes. Mosquito defensins (100 μM) and JEV (10 MID_{50}) were premixed, incubated at 4°C for 2 h, and inoculated into female mosquitoes. JEV E mRNA levels in the whole body at 7 and 10 days after JEV infection were quantified by real-time PCR. (B) The efficiency of defensin knockdown via RNAi *in vivo*. siRNAs targeting total *C. pipiens pallens* defensins or defensin A were injected into mosquitoes, which were collected at 3 days after injection and used to detect defensin mRNA levels by real-time

(Continued on next page)

TABLE 3 IC_{50} measurement

Defensin	Cell	IC_{50} value (μM)[a]
Aedes albopictus defensin C	C6/36	2,369.342
Aedes albopictus defensin C	Vero	2,311.063
Aedes albopictus defensin C	BHK-21	2,803.65
Human defensin β2	C6/36	7,151.357
Human defensin β2	Vero	3,849.642
Human defensin β2	BHK-21	4,427.204

[a]IC_{50} value was measured by using MTT methods. Six concentration gradients were performed on each cell.

onstrating that defensins from mosquitoes or humans have diverse functions in JEV infection on C6/36 cells (Fig. 2G, i and ii).

The above-described data provided contradictory but interesting findings regarding mosquito defensins. To expand the scope of our analysis of mosquito defensins, we introduced human defensin β2 for comparative analysis in this study. Human defensin β2 is a mammalian defensin with high antiviral activity. In addition, human defensin β2 shows 100% homology with *Macaca mulatta* beta-defensin 4A (GenBank accession number Q9BDS9.1). We compared the effects of mosquito and mammalian defensins on the mammalian cell lines A549 (*Homo sapiens*), Vero (*M. mulatta*), and BHK-21 (*Mus musculus*). First, we detected the effects of defensins in A549 and Vero cells. *A. albopictus* defensin C reduced JEV replication by 1.96- to 2.25-fold (Fig. 2H, i and Fig. 2I, i) and decreased JEV $TCID_{50}$ levels by 0.7- to 0.8-fold (Fig. 2H, ii and Fig. 2I, ii), indicating that it inhibits JEV infection in mammalian cells when administered at the same dose as human defensin. Although the inhibition ability of *A. albopictus* defensin C was lower than that of human defensin β2 in both A549 (Fig. 2H, i, $P < 0.01$) and Vero (Fig. 2I, i, $P < 0.05$) cells, *A. albopictus* defensin C still inhibited JEV replication. BHK-21 cells were also used as a mammalian cell model without human defensin β2 in which to analyze defensins. The results showed that mosquito defensin had an effect on JEV infection similar to that of human defensin (Fig. 2J, i and ii). Therefore, the effects of mosquito defensin in facilitating JEV apply to only mosquitoes and mosquito cells. To confirm that the effect of defensins was not due to cytotoxicity, we measured the 50% infective concentration (IC_{50}) of each defensin through MTT assays. The results showed that defensins had no significant cytotoxic effect on cells (Table 3).

Mosquito defensin enhances JEV adsorption to target cells. To study the exact mechanisms by which mosquito defensin facilitates JEV infection, we analyzed the influence of *A. albopictus* defensin C at different steps of infection in C6/36 cells. As the steps of infection can be measured by temperature and time, we detected the adsorption, uncoating, and replication of JEV (27). To assess binding, virus and defensin were mixed and inoculated into C6/36 cells on ice, which were incubated for 4 h, washed with cold phosphate-buffered saline (PBS) five times, and cultured at 28°C for 48 h in fresh medium. To assess uncoating, the virus was inoculated into C6/36 cells on ice, which were incubated for 4 h and washed with PBS five times. Fresh medium containing defensin was added to the cells, which were then incubated for 6 h. After

FIG 2 Legend (Continued)
PCR. (C) Mosquito defensin knockdown inhibited JEV infection in *C. pipiens pallens* mosquitoes. Female mosquitoes were injected with siRNAs and transfected for 3 days, after which JEV was injected at a dose of 10 MID_{50}. JEV E mRNA levels in the whole body at 6 days after JEV infection were quantified by real-time PCR. (D) The efficiency of defensin knockdown via RNAi *in vitro*. siRNAs targeting *Ae. albopictus* defensins were transfected into C6/36 cells for 24 h. The cells were collected, and defensin mRNA was measured by real-time PCR. (E) Defensin knockdown inhibited JEV infection in C6/36 cells. siRNAs targeting defensin were transfected into C6/36 cells for 24 h, after which JEV (MOI of 0.1) was inoculated into C6/36 cells without changing the medium. The cells and supernatant were collected at 2 days after infection to quantify JEV E mRNA levels (i) and the $TCID_{50}$ (ii). (F) Mosquito defensins facilitated JEV infection in C6/36 cells, as shown by IFA detection. Mosquito defensins (50 μM) and JEV (0.5 MOI) or mock were premixed at 4°C and inoculated into C6/36 cells for 2 h (upper three panels). siRNAs targeting defensin were transfected into C6/36 cells for 24 h, and JEV or mock was inoculated into C6/36 cells for 2 h (lower three panels). An IFA of the cells was performed at 3 days after infection. Bar, 10 μm. JEV E MAb used in IFA analysis was validated in a previous study (18). (G) Mosquito defensins facilitated JEV infection in C6/36 cells. Defensins (50 μM) and JEV (0.5 MOI) were premixed at 4°C and inoculated into C6/36 cells for 2 h. The cells and supernatant were collected at 3 days after infection to quantify JEV E mRNA levels (i) and the $TCID_{50}$ (ii). (H to J) Mosquito defensins inhibited JEV infection in mammalian cells. Mosquito defensins (50 μM) and JEV (MOI of 0.5) were premixed at 4°C and inoculated into A549 (H), Vero (I) or BHK-21 (J) cells for 1.5 h at 37°C. The cells and supernatants were collected at 48 h after infection and used to quantify JEV E mRNA levels (i) and the $TCID_{50}$ (ii). All experiments were performed in triplicate at least three times. Data are shown as the mean values ± standard deviations.

incubation, the cells were washed and cultured for another 42 h in new medium. To assess replication, the virus was inoculated into C6/36 cells on ice, which were incubated for 4 h and washed with PBS five times. New medium without defensin was added to the cells, which were incubated for 48 h, and defensin was added to the medium after 6 h of culture. The cells were collected and used to quantify JEV E mRNA levels by real-time PCR. Adsorption was determined to be a key step in the effect of mosquito defensin in facilitating JEV (Fig. 3A). Next, we detected JEV adsorption at different time points. JEV mixed with defensin or scrambled peptides was inoculated into C6/36 cells, which were incubated for different periods on ice. After being washed with PBS three times, cells to which JEV had adsorbed were collected. JEV E mRNA levels were determined by real-time PCR. C6/36 cells treated with *A. albopictus* defensin C showed significantly higher JEV E mRNA levels at 4 and 6 h after adsorption, demonstrating that defensin enhanced JEV adsorption to C6/36 cells (Fig. 3B). Analysis by IFA showed that JEV adsorption greatly increased over the time course of *A. albopictus* defensin C treatment (Fig. 3C and D). Both nuclear staining (DAPI) and membrane staining (Did) of C6/36 cells were conducted for IFA absorption analysis. Stronger JEV adsorption was observed for the *A. albopictus* defensin C groups than the control groups at each time point by both DAPI staining (Fig. 3C) and Did staining (Fig. 3D) cells. To study how mosquito defensin facilitates JEV adsorption, fluorescein isothiocyanate (FITC)-labeled *A. albopictus* defensin C was used. Defensin C-FITC and JEV were mixed before incubation at 0°C. After incubation, unabsorbed defensin and JEV were removed by washing with PBS five times. The cells were collected at the indicated time points and used to observe the colocalization of defensin and JEV. Strong colocalization between *A. albopictus* defensin C and JEV was observed on the cell surface (Fig. 3E), and this colocalization increased over time. Thus, the effect of mosquito defensin in facilitating JEV can be attributed to their binding. Additionally, JEV mixed with *C. pipiens pallens* defensin A showed a high adsorption capacity in the salivary glands (Fig. 3F). Taken together, our results indicate that mosquito defensin can bind JEV and facilitate virus adsorption.

The interaction between defensin and JEV was also confirmed by enzyme-linked immunosorbent assay (ELISA). The wells of a plate were coated with *A. albopictus* defensin C, incubated with JEV, and then incubated with anti-JEV antibody. As expected, JEV bound defensin efficiently. Even when 250 ng of defensin was used to coat the wells, the JEV level was significantly higher than that in the control group (Fig. 3G). To determine the adsorption capacity of the JEV-defensin complex to C6/36 cells, we coated the wells of a plate with fresh C6/36 cells after polylysine treatment, added premixed defensin and JEV, and detected JEV with anti-JEV monoclonal antibody (MAb). In accordance with the results of real-time PCR and analysis by IFA, the interaction of defensin with JEV significantly enhanced JEV adsorption to C6/36 cells (Fig. 3H).

Based on the previous results, we deduced that mosquito defensin can efficiently bind the cell surface. To confirm the interaction between mosquito defensin and C6/36 cells, FITC conjugated defensin peptides were used and followed by a fluorescent ELISA. The interaction of mosquito defensin with the cell surface was assessed through FITC-ELISA (excitation, 488 nm). Defensin directly interacted with C6/36 cells, and a higher FITC value than that of the control was observed in different cell densities (Fig. 3I). This finding implies that the effect of defensin in facilitating JEV is caused by the increased affinity of JEV for the cell surface.

Defensin directly binds the JEV ED III domain. Defensins can bind viral E proteins (25). To precisely understand how the interaction of JEV with defensin mechanistically enhances JEV infection, we expressed the three structural proteins of JEV (C, prM, and E) and the exposed region of the E protein (the ED III domain) in the S2 insect protein expression system (28, 29) and further purified these proteins via 6×His agarose. Specificity of MAbs against JEV prM and C proteins was validated, respectively (Fig. 4A and B). JEV E MAb was performed as described previously (18). To analyze the

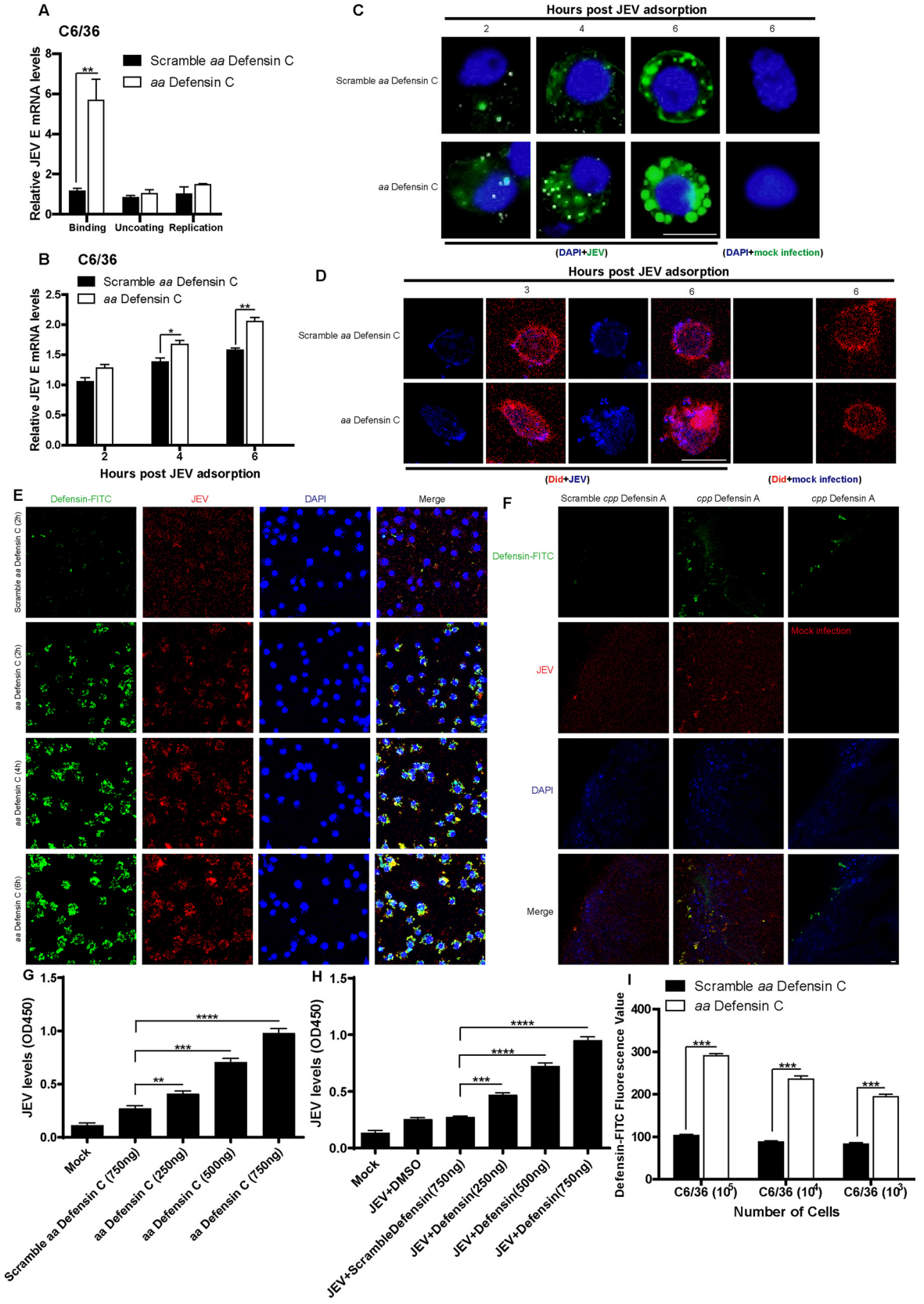

FIG 3 Mosquito defensin facilitated JEV adsorption to mosquito cells. (A) The steps of JEV infection in C6/36 cells. The effects of different treatments on the different steps of JEV infection were analyzed in C6/36 cells. To assess binding, virus (MOI of 0.5) and defensin (50 μM)

(Continued on next page)

interaction between viral proteins and defensins, two ELISA methods were used. The wells of a plate were coated with defensin, incubated with purified proteins, and assessed with the corresponding antibodies. Scrambled defensin was used as a control. The absorbance values showed high affinity between *A. albopictus* defensin C and the E protein or the ED III domain of the E protein; specifically, these affinities were ~0.95 and ~1.17, respectively (Fig. 4C). Consistent results were observed by detection of defensin-FITC. The wells of a plate were coated with purified viral proteins and then incubated with *A. albopictus* defensin C-FITC or scrambled defensin-FITC. The E protein and ED III domain showed high fluorescence values of 332 and 369, respectively (Fig. 4D). The results of both tests suggest that the ED III domain of the E protein is the key region involved in binding between *A. albopictus* defensin C and JEV. Subsequently, purified E protein and the ED III domain were individually mixed with *A. albopictus* defensin C and used to inoculate C6/36 cells at 0°C for 4 h. Unabsorbed defensin and JEV after incubation were removed by washing with PBS. The effects of *A. albopictus* defensin C in facilitating E protein and ED III domain adsorption were observed by fluorescence microscopy (Fig. 4E and F). The E protein and ED III domain bound more efficiently to C6/36 cells in the presence of *A. albopictus* defensin. Additionally, *A. albopictus* defensin C individually colocalized with the E protein and ED III domain on C6/36 cells (Fig. 4E, merge panel). The same results were observed in membrane-stained C6/36 cells. The E protein and ED III domain bound more efficiently to the C6/36 cell surface in the presence of *A. albopictus* defensin C, and *A. albopictus* defensin C also individually colocalized with the E protein and ED III domain on the C6/36 surface (Fig. 4F). These findings indicate that the ED III domain of the JEV E protein is responsible for the binding of JEV with *A. albopictus* defensin C.

LRP2 is responsible for mosquito defensin-mediated JEV adsorption. As an extracellular protein, defensin has been reported to interact with receptors on the cell surface, consequently affecting intracellular signaling networks. To define the relationship between defensin/cell-surface receptors and enhanced adsorption, we analyzed cell-surface receptors that interact with defensin. We knocked down the expression of a series of potential receptors on the cell surface through RNAi and found that LRP2 is responsible for defensin binding (30, 31). The results indicated that the binding of mosquito defensin to cells was decreased after LRP2 knockdown (Fig. 5A to C). LRP2 interference harmed the interaction between defensin and C6/36 cells, indicating that LRP2 is related to the adsorption of extracellular defensin. We further studied the role of LRP2 in JEV adsorption mediated by defensin. Based on the results following significant RNA knockdown (Fig. 5A), no difference in JEV adsorption was observed

FIG 3 Legend (Continued)
were mixed and inoculated into C6/36 cells, which were incubated on ice for 4 h, washed with PBS five times, and cultured for 48 h in new medium. To assess uncoating, virus (MOI of 0.5) was inoculated into C6/36 cells on ice for 4 h, after which the cells were washed with PBS five times. Fresh medium containing defensin (50 μM) was added to the cells, which were incubated for 6 h. After incubation, the cells were washed and cultured for another 42 h in new medium. To assess replication, virus (MOI of 0.5) was inoculated into C6/36 cells, which were incubated on ice for 4 h and washed with PBS five times. New medium without defensin was added to the cells, which were incubated for 48 h, and defensin (50 μM) was added to the medium after 6 h of culture. The cells were collected and used to quantify JEV E mRNA levels by real-time PCR. (B) *A. albopictus* defensin C facilitated JEV adsorption to C6/36 cells in a time-dependent manner. *A. albopictus* defensin C (50 μM) and JEV (0.5 MOI) were premixed at 4°C and inoculated into C6/36 cells on ice for 4 h. Unabsorbed JEV was removed by washing with PBS three times. The cells were collected and used to quantify JEV E mRNA levels by real-time PCR. (C and D) IFA analysis of JEV adsorption to C6/36 cells. *A. albopictus* defensin C-FITC (50 μM) and JEV (1 MOI) were premixed at 4°C and inoculated into C6/36 cells on ice. Unabsorbed JEV and defensin were removed by washing with PBS three times. The cells were stained with antibody and DAPI (C) or Did (D). (E) Colocalization of defensin and JEV on the cell surface. *A. albopictus* defensin C-FITC (50 μM) and JEV (1 MOI) were premixed at 4°C and inoculated into C6/36 cells on ice for 2 h, 4 h, and 6 h. Unabsorbed JEV and defensin were removed by washing with PBS three times. The cells were treated to observe JEV E (red fluorescence), defensin-FITC (green fluorescence), and nuclei (blue fluorescence). Bar, 10 μm. (F) Colocalization of *C. pipiens pallens* defensin A-FITC and JEV in the salivary gland. The salivary glands from uninfected female mosquitoes were freshly isolated. Premixed *C. pipiens pallens* defensin A-FITC (50 μM) and JEV (1 MOI) were added to the salivary glands, which were incubated at room temperature for 1 h. JEV E was labeled with a monoclonal antibody (red fluorescence). Defensin-FITC was detected as green fluorescence, and nuclei were stained with DAPI (blue fluorescence). Bar, 20 μm. (G) JEV binds defensin. Wells in a plate were coated with *A. albopictus* defensin C or DMSO, which was incubated with JEV or mock, followed by assessment with anti-JEV monoclonal antibody. (H) A mixture of defensin and JEV binds C6/36 cells. After polylysine treatment, the wells in a plate were coated with C6/36 cells to which premixed defensin and JEV were added, and detection was performed with anti-JEV antibody. (I) Defensin binds C6/36 directly. The polylysine-treated wells were coated with C6/36 cells to which defensin-FITC was added, and the fluorescence was detected. All experiments were performed in triplicate at least three times. Data are shown as the mean values ± standard deviations.

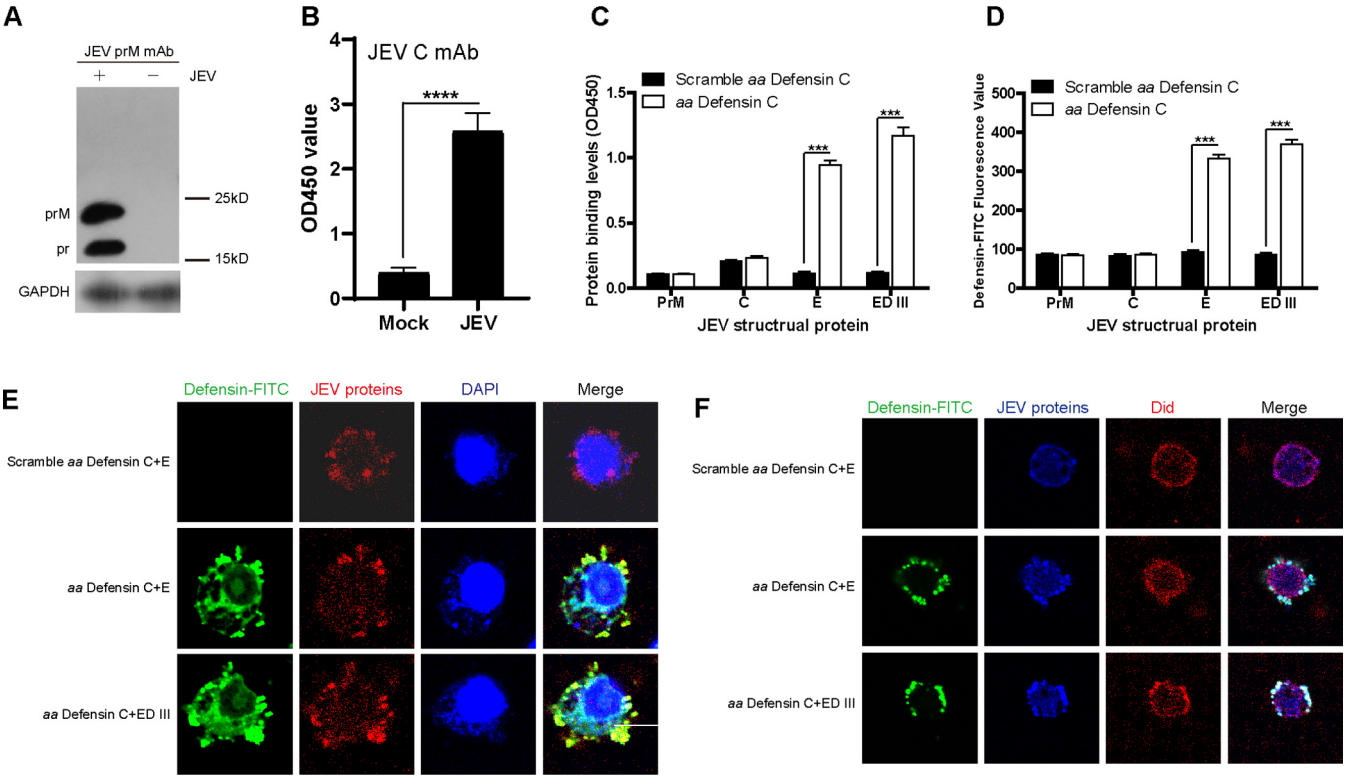

FIG 4 Mosquito defensin bound JEV virions. (A and B) Specifics of JEV prM and C monoclonal antibody. prM MAb was validated by Western blot analysis. BHK cell samples mock infected or infected with JEV were used (A). C MAb was validated by the indirect ELISA method. Supernatant of BHK cells infected with JEV or mock infected was used for coating (B). (C) Viral proteins bind mosquito defensin. Wells in a plate were coated with *A. albopictus* defensin C and incubated with JEV structural proteins. Rabbit polyclonal antibodies against C protein and mouse monoclonal antibodies against the prM and E proteins and ED III domain were utilized to detect viral protein binding. (D) Mosquito defensin-FITC binds viral proteins. Wells in a plate were coated with purified JEV structural proteins, which were incubated with *A. albopictus* defensin C-FITC. The fluorescence value for each well was measured. (E and F) Colocalization between defensin and the E protein or ED III domain. Defensin-FITC and the E protein or ED III domain were premixed at 4°C and inoculated into C6/36 cells, which were incubated on ice for 4 h. Unabsorbed defensin and proteins were removed by washing with PBS three times. (E) The cells were stained with monoclonal antibody and DAPI to observe JEV E (red fluorescence), defensin-FITC (green fluorescence), and nuclei (blue fluorescence). (F) The cells were stained with monoclonal antibody and Did to observe JEV E (cyan fluorescence), defensin-FITC (green fluorescence), and the membrane (red fluorescence). Bar, 10 μm. All experiments were performed in triplicate at least three times. Data are shown as the mean values ± standard deviations.

between cells with or without LRP2 interference were infected with JEV alone (Fig. 5D). These results implied that LRP2 does not impact JEV infection directly. However, when C6/36 cells were incubated with defensin and JEV, the JEV level was lower in LRP2-knockdown cells. The JEV mRNA level in LRP2-knockdown cells was 2.8-fold lower than that of the group transfected with scramble (negative control, NC) siRNA (Fig. 5D), and the $TCID_{50}$ level was significantly lower in LRP2-knockdown cells than in NC siRNA-transfected cells (Fig. 5E). Additionally, the results of IFA analysis were in accordance with the above-mentioned findings. JEV fluorescence in LRP2-knockdown cells was lower than that of NC siRNA-transfected cells (Fig. 5F). In *in vivo* experiments, mosquitoes were inoculated with LRP2 or NC siRNA for 3 days (Fig. 5G) and then inoculated with a mixture of defensin and JEV. Whole-mosquito samples were collected at 3 days after infection. The JEV mRNA level was significantly lower in the LRP2-knockdown group than in the NC group (Fig. 5H), thus indicating that LRP2 participates in defensin-mediated virus adsorption. Taken together, our findings indicate that LRP2 is the cell-surface factor responsible for defensin-mediated JEV adsorption. LRP2/defensin constitutes an axis that mediates JEV adsorption in mosquitoes. Lipoprotein receptor-related protein 4 (LRP4) and CXCR4 also showed binding activity with defensin, but this activity did not influence JEV adsorption (data not shown).

Mosquito defensins facilitate JEV dissemination in the salivary gland. To assess the enhanced transmission potential of JEV mediated by mosquito defensins, we detected virus levels within the salivary gland of defensin-treated mosquitoes (32, 33).

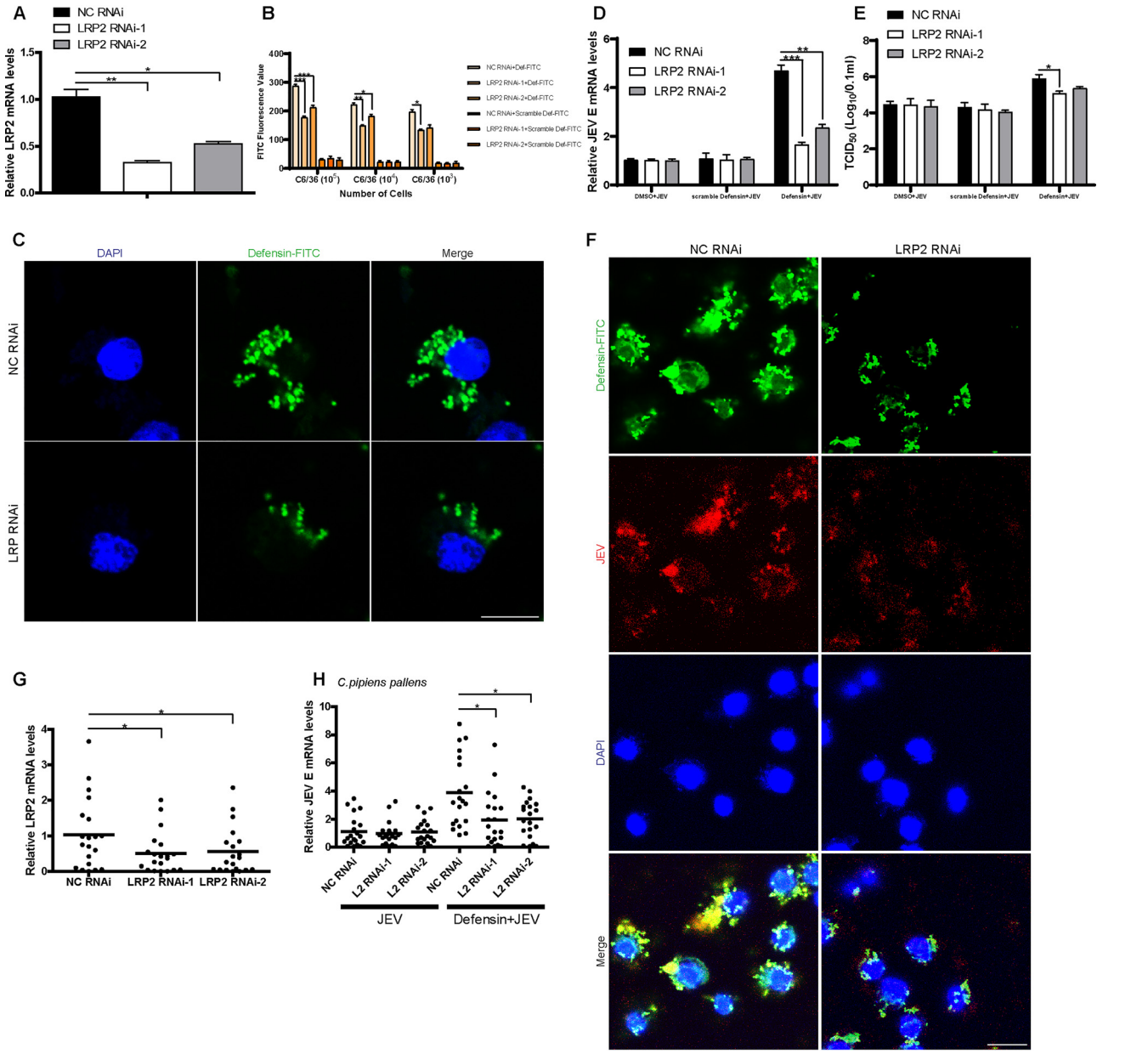

FIG 5 The LRP2/defensin pathway mediates JEV adsorption. (A) The efficiency of LRP2 knockdown via RNAi *in vitro*. LRP2 siRNA targeting *A. albopictus* LRP2 was transfected into C6/36 cells for 24 h. The cells were collected, and LRP2 mRNA was measured by real-time PCR. (B and C) Defensin adsorption was influenced by LRP2. Polylysine-treated wells in a plate were coated with C6/36 cells, which were transfected with LRP2 siRNAs. Defensin-FITC was inoculated into the cells at 24 h after transfection. After incubation on ice for 2 h, unabsorbed defensin was removed by washing with PBS three times. The fluorescence was detected with a fluorescence analyzer (B) or fluorescence microscope (C). (D, E, and F) JEV adsorption on C6/36 cells was influenced by the LRP2/defensin pathway. Polylysine-treated wells in a plate were coated with C6/36 cells, which were transfected with LRP2 siRNAs. Premixed JEV and *A. albopictus* defensin C were inoculated into the cells at 24 h after siRNA transfection. After incubation at room temperature or on ice for 2 h, unabsorbed defensin and virus were removed by washing with PBS three times. For real-time PCR (D) and $TCID_{50}$ (E) measurements, C6/36 cells and supernatants were collected at 2 days after infection. For IFA analysis, C6/36 cells were treated immediately after inoculation on ice (F). JEV E was labeled with monoclonal antibody (red fluorescence). Defensin-FITC was detected as green fluorescence, and nuclei were stained with DAPI (blue fluorescence). Bar, 10 μm. (G) The efficiency of LRP2 knockdown via RNAi *in vivo*. siRNA targeting *C. pipiens pallens* LRP2 was injected into mosquitoes, which were collected at 3 days after injection and used to detect LRP2 mRNA levels by real-time PCR. (H) *In vivo* JEV adsorption was influenced by the LRP2/defensin pathway. Three days after mosquitoes were injected with LRP2 siRNA, the mosquitoes were injected with premixed JEV and defensin. Six days after infection, the mosquitoes were collected and used to detect JEV E mRNA levels in the whole body. All experiments were performed in triplicate at least three times. Data are shown as the mean values ± standard deviations.

Both microinjection and blood meal were used in this experiment. JEV and mosquito defensin were mixed before inoculation. Mosquitoes injected with JEV and defensin peptide were collected at 7 or 10 days after infection. Fresh salivary glands were isolated and assessed by using real-time PCR. JEV levels were significantly increased in

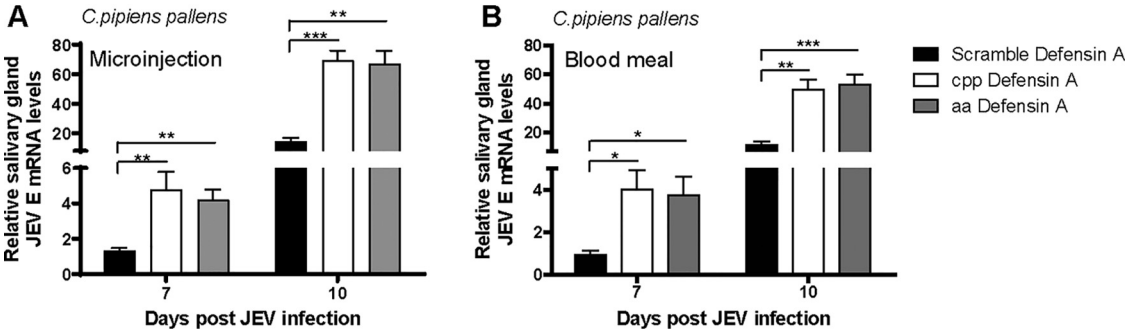

FIG 6 Mosquito defensin enhanced JEV replication in the salivary gland. (A) JEV E mRNA levels within the salivary gland following microinjection. *C. pipiens pallens* defensin A (100 μM) and JEV (10 MID_{50}) were premixed, incubated at 4°C for 2 h, and injected into female mosquitoes. Salivary glands were isolated at 7 and 10 days after injection and used to detect JEV E mRNA levels by real-time PCR. (B) JEV E mRNA levels within the salivary gland following a blood meal. *C. pipiens pallens* defensin A (100 μM) and JEV (10^3 MID_{50}) were premixed at 4°C. The mixture was added to fresh blood with anticoagulant, and the mosquitoes were fed a blood meal for 2 h. Their salivary glands were isolated at 7 and 10 days after infection and used to detect JEV E mRNA levels by real-time PCR. All experiments were performed in triplicate at least three times. Data are shown as the mean values ± standard deviations.

the *C. pipiens pallens* defensin- and *A. albopictus* defensin-treated groups (Fig. 6A). The salivary gland JEV level in the *C. pipiens pallens* defensin-treated group was 3.5-fold higher at day 7 after infection and 3.1-fold higher at day 10 after infection than that of the scramble-treated group. *A. albopictus* defensin was shown to play the same role as *C. pipiens pallens* defensin. We further employed infection via blood meal to measure the effect of defensin in JEV distribution in the mosquito salivary gland. Five-day-old female mosquitoes were deprived of sucrose and water for 24 h prior to the blood meal. The mosquitoes were then fed blood infected with JEV containing defensin peptides for 2 h. Fresh blood was collected from healthy mice and delivered through a Hemotek membrane feeding apparatus. Two milliliters of blood containing JEV (5×10^6 $TCID_{50}$) and peptide (200 μM) was used for each group. The salivary gland JEV level in the *C. pipiens pallens* defensin-treated group at days 7 and 10 after infection was 4.2-fold higher than that of the scramble-treated group (Fig. 6B). JEV levels in the *A. albopictus* defensin-treated group were also higher than those in the scramble-treated group. These results imply that mosquito defensins facilitate JEV dissemination in mosquitoes and increase transmission potential after infection.

DISCUSSION

JEV is a serious mosquito-borne disease common in tropical and subtropical regions of Asia and the Pacific (2, 9, 10). A large number of people are at risk of JEV, which can cause death or permanent sequelae. Pigs serve as the reservoir host of the virus, and humans, horses, and other animals are dead-end hosts. Mosquitoes, especially *Culex* mosquitoes, are the most important vector for JEV (4). At present, the prevention and control of JEV mainly relies on vaccine immunization, which confers protection over a limited duration. JEV remains a threat to the health and even life of immunocompromised individuals, children, and older people (6, 7). With the problem of increasing global warming, the clinical incidence of JEV is increasing (34). Few mechanistic studies have focused on JEV transmission by mosquito vectors. Therefore, understanding the interaction between JEV and mosquito vectors and the mode of JEV immune escape is of practical importance in controlling this mosquito-borne disease.

In this study, we analyzed the gene expression of defensins from *C. pipiens pallens* and *A. albopictus*. Defensin A and an unnamed defensin from *C. pipiens pallens* and defensins A, B, and C from *A. albopictus* shared high sequence similarity, implying the similar functions of these defensins. Subsequently, we confirmed that defensin A and defensin C are the dominant types of defensin expressed in *C. pipiens pallens* and *A. albopictus*, respectively. Based on their high-amino acid sequence homology, we synthesized *C. pipiens pallens* defensin A and *A. albopictus* defensin C for use in further

studies. The nucleotide sequence of *C. pipiens pallens* defensin A (GenBank accession number MH756645) has been released in the NCBI database.

The upregulation of defensin after JEV infection is consistent with reports on other flaviviruses (35). The most pronounced increase in defensin was observed at 7 days after infection. From the whole-organism perspective, defensin was upregulated to a greater degree in the salivary gland and whole body than in the midgut. Viral load in the midgut showed a lower level than that in other organs in several studies (17). Maybe using microinjection will affect the infection route of virus and then affect the viral load in mosquito. JEV replication in the salivary gland, the tissue most sensitive to JEV (18), was positively correlated with defensin levels.

The mature defensin protein was utilized to study the role of mosquito defensin in JEV infection (25, 36). In general, defensins are fewer than 60 amino acids in length and processed from a precursor protein. Mosquito defensin and human defensin β2 are composed of 40 amino acids and 34 amino acids (37), respectively, and were found to exhibit low sequence similarity. Unexpectedly, mosquito defensin, unlike human defensin, was shown to facilitate JEV infection, but its effect in facilitating JEV infection was observed in only mosquito cells or mosquitoes. Thus, JEV utilizes the host defense system, reflecting its "cunning" nature in infection (38). However, mosquito defensin inhibited JEV infection in mammalian cells, indicating its varied mechanisms of action and the complicated interaction between virus and host (19).

Further analysis demonstrated that mosquito defensin facilitated JEV adsorption to target cells by directly binding JEV virions (27, 39). By screening JEV structural proteins, we found that mosquito defensin bound the ED III domain of the JEV E protein. The weak antiviral effect of mosquito defensin against JEV is likely related to its binding to the viral E protein (Fig. 2H to J) (19, 25). Because mosquito defensin facilitated JEV infection, the binding of defensin and the ED III domain suggest that mosquito defensin has only weak antiviral effects. Nevertheless, this binding enhanced virion adsorption to a large extent. The broad transmission of JEV by mosquitoes is ascribed to both the simple immune system of mosquitoes and the infection strategy of the virus. Mosquito defensin could improve the adsorption of JEV on target cells.

Defensin receptors expressed on the cell surface may lead to enhanced adsorption. We screened potential cell-surface receptor proteins that bind defensin through RNAi and found that the LRP2-defensin axis is responsible for JEV adsorption. In mammalian animals, LRP2 is a defensin receptor that regulates the contraction of smooth muscle cells by binding human alpha defensin (30, 31). However, the roles of LRP2 in mosquitoes have not been reported. In the present study, we demonstrated that LRP2 participates in JEV adsorption mediated by defensin. JEV first binds defensin, and then, owing to the affinity of defensin for LRP2, the defensin/JEV complex more readily adsorbs to the cell surface, thereby increasing the possibility of infection. This proposed mechanism by which defensin/LRP2 promote JEV infection is similar to that by which C-type lectin/PTP-1 promotes JEV and West Nile virus (WNV) infection. That is, viruses first bind extracellular secreted proteins with a high cellular affinity and then bind cell-surface receptors to infect target cells.

JEV infection upregulated mosquito defensin expression in the salivary gland, and defensin also increased the JEV level in the salivary gland, suggesting that defensin may influence the transmission of JEV by mosquitoes. Therefore, we detected the influence of mosquito defensin on JEV transmission potential. Microinjection and infection via blood meal were used to analyze the JEV level in the mosquito salivary gland, which represents the transmission potential of JEV in mosquitoes (32). As expected, mosquito defensin enhanced the JEV level in the mosquito salivary gland, suggesting that the interaction between defensin and JEV within mosquito regulates mosquito-borne JEV transmission.

Antimicrobial peptides are important immune effectors in insects. And fast defensin response after microorganism infection in mosquito was well defined in a published report (40). In natural conditions, JEV establishes midgut infection first. This local infection will induce rapid upregulation and extracellular secretion of defensin, and

then the extracellular defensins are likely to enhance JEV infection in other tissues and dissemination in the salivary gland by increasing virus binding.

To the best of our knowledge, this is the first report of the effects of mosquito defensin on JEV infection in mosquito vectors, revealing a new immune escape mechanism in JEV infection and transmission. This study broadens our knowledge of the transmission of JEV as well as other mosquito-borne viruses, providing novel insights into viral transmission mechanisms.

MATERIALS AND METHODS

Ethics statement. All animal experiments were performed in compliance with the Guidelines on the Humane Treatment of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China, policy number 2006 398) and were approved by the Institutional Animal Care and Use Committee at the Shanghai Veterinary Research Institute (IACUC number Shvri-Pi-0124).

Cells, defensin, antibodies, and viruses. Baby hamster kidney (BHK-21) and African green monkey kidney (Vero) cells were purchased from the ATCC (Rockville, Maryland) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. C6/36 cells (ATCC) were cultured in RPMI 1640 medium supplemented with 10% FBS at 28°C.

Mature *C. pipiens pallens* defensin A (NCBI accession number MH756645), A. albopic*tus* defensin C (XP_019527114.1), human defensin β2 (NP_004933.1), and scrambled defensin peptides (purity $\geq$99%) were synthesized by WC-Gene Biotech Ltd. (Shanghai, China). The amino acid sequences are shown in Table 2. The defensins were dissolved in DMSO (for cell, *ex vivo*, and *in vivo* experiments) or PBS (for ELISA detection) and stored at room temperature. Defensins labeled with FITC were kept in the dark at room temperature.

Mouse monoclonal antibody against JEV structural proteins was prepared and stored in our laboratory. prM MAb can be used in Western blot, immunofluorescence, and ELISA experiments. C MAb can be used in immunofluorescence and ELISA experiments. E MAb can be used in coimmunoprecipitation Western blot, immunofluorescence, and ELISA experiments.

JEV strain N28 (NCBI accession number: GU253951.1) was stored in our laboratory and propagated in C6/36 cells. The $TCID_{50}$ and MID_{50} of the virus were measured in BHK-21 cells or female mosquitoes and calculated using the Reed-Muench method (17, 41).

Infection and RNA interference *in vitro*. Defensins or scrambled defensin peptides were premixed with JEV (multiplicity of infection [MOI] = 0.1) at 4°C and then inoculated into cells. C6/36 cells were incubated at 28°C for 2 h. Vero and BHK-21 cells were incubated at 37°C for 2 h. At 24 to 120 h postinfection, the supernatant or cells were collected. Viral titer was determined by the $TCID_{50}$ method, and mRNA expression levels were measured by real-time PCR. To determine JEV adsorption, defensins were premixed with JEV at 4°C for 2 h. C6/36 cells were incubated with the mixture on ice for different times. Unabsorbed JEV was removed by washing with PBS three times. The cells were collected for JEV E mRNA quantification or other measurements.

For the *in vitro* RNA interference, siRNA (Table 1) was transfected into C6/36 cells with Cellfectin II reagent (Invitrogen). JEV was inoculated at 24 h posttransfection. At 72 h postinfection, the cells were collected. Total RNA was isolated, and the viral or gene load was determined by real-time PCR.

Infection and RNA interference *in vivo*. For *in vivo* experiments, 10-fold serial dilutions were made from a $10^{9.3}$ $TCID_{50}$ JEV stock. Cold-anesthetized 5-day-old female mosquitoes were randomly divided into various groups ($n \geq 13$). Both the microinjection and blood meal methods were carried out in the infection experiment. For microinjection, the mosquitoes were infected by microinjection (250 nl) into the thorax. An Eppendorf CellTram oil microinjector and 15-μm needles were used for injecting the mosquitoes. Control mosquitoes were injected with an equivalent volume of PBS (17, 18, 42). The mosquitoes were harvested, and the viral loading was quantified. For blood meal, fresh blood of a specific-pathogen-free mouse was collected in tubes with anticoagulant. Virus or defensin peptides were mixed and added into fresh blood before feeding. Then, 2 ml blood was used in the blood meal with a Hemotek FU1 feeder for each group (32, 43).

In vivo RNAi was performed as described previously (18). The siRNA targeting the *C. pipiens pallens* genes was synthesized by Genepharma (Shanghai, China). The sequences are shown in Table 1. For RNAi and virus challenge, female mosquitoes at 5 days after eclosion were injected into the thorax with 2 μg dsRNA in 250 nl PBS. After a 3-day recovery period, the mosquitoes were microinjected with JEV at different MID_{50} in 250 nl PBS for functional studies.

RNA isolation and real-time PCR. For real-time PCR, RNA was extracted from cell suspensions or mosquito samples with a Qiagen total RNA isolation kit according to the manufacturer's instructions. The RNA concentration was measured with a NanoDrop spectrophotometer. cDNA was generated with an RT Master reverse transcription kit (TaKaRa) according to the manufacturer's instructions. Real-time quantitative PCR experiments were performed in an ABI Prism 7500 sequence-detection system (Applied Biosystems, Foster City, CA) with SYBR green PCR Master Mix (TaKaRa) according to the manufacturer's instructions. The primer sequences are listed in Table 1. The thermal cycling conditions were as follows: 10 min at 95°C, followed by 40 cycles of 95°C for 5 s and 60°C for 1 min. All experiments were performed in triplicate, and gene expression levels are presented relative to those of β-actin. For the comparative analysis of gene expression, fold changes of each gene were

calculated. The fold change in relative gene expression compared with the control was determined with the standard $2^{-\Delta\Delta CT}$ method.

Virus titer. Supernatants were harvested from cell cultures for $TCID_{50}$ assays as described previously (18). Briefly, BHK-21 cells were seeded on a 96-well plate and grown to 60% confluence. The supernatants were diluted in a 10-fold dilution series and added to each well of the 96-well plate. One hundred microliters of each dilution was added in eight replicate wells, and eight replicate mock controls were set. The plates were incubated at 37°C for 1.5 h. Then, the supernatants were discarded and replaced with 100 μl of DMEM supplemented with 1% FBS. After 5 days in culture, the cytopathic effect was recorded. The $TCID_{50}$ of the virus was calculated by the Reed-Muench method (41).

Indirect immunofluorescence and Western blotting. Indirect immunofluorescence and Western blotting were performed as described previously (18). The antibodies used were mouse anti-JEV E monoclonal antibody, rabbit anti-mosquito β actin polyclonal antibody, goat anti-mouse IgG-horseradish peroxidase (HRP) antibody (1:10,000; Santa Cruz), Alexa Fluor 405-conjugated anti-mouse IgG antibody (1:500; Abcam), Alexa Fluor 488-conjugated anti-rabbit IgG antibody (1:500; Thermo Fisher Scientific), and Alexa Fluor 594-conjugated anti-rabbit IgG antibody (1:500; Thermo Fisher Scientific). DAPI and Did were used for nucleus and membrane staining. Immunofluorescence was imaged with a Nikon C1Si confocal laser scanning microscope.

For tissue immunofluorescence assays, salivary glands were isolated on sialylated slides, washed with PBS, fixed with 4% paraformaldehyde for 1 h, and blocked in PBS with 2% bovine serum albumin (BSA) at room temperature for 2 h. The samples were incubated with a mixture of JEV and *C. pipiens pallens* defensin A-FITC, detected with mouse anti-JEV E monoclonal antibody and imaged with a Nikon C1Si confocal laser scanning microscope.

Protein expression and ELISA. The purified JEV structural proteins (C, M, E, ED III) from the S2 insect expression system (Invitrogen) were quantified by using the bicinchoninic acid (BCA) assay. Expressed proteins were used for ELISA or IFA analysis (44).

For defensin ELISA, defensin peptide was dissolved in PBS and then was diluted with 0.1 M dicarbonate (pH 9.6) to a final concentration of 250 to 750 ng. The plate was coated overnight and incubated with 2% BSA for 2 h. Afterward, 100 μl JEV virus (1×10^5 $TCID_{50}$) was added and incubated for 30 min at room temperature. The wells were washed with PBST five times, and mouse polyclonal antibody to JEV was added to the wells and incubated for 30 min. The wells were washed with PBST five times, and goat anti-mouse antibody labeled with HRP was added. After incubation at room temperature for 30 min and washing with PBST five times, TMB was added to the wells as a chromogenic substrate. The plate was developed in the dark for 10 min, and H_2SO_4 was added to stop the reaction. The absorbance of each well was read at 450 nm.

For viral protein ELISA, purified JEV structural proteins diluted in 0.1 M dicarbonate (pH 9.6) were added to the plate wells. The plate was coated overnight and incubated with 2% BSA for 2 h. Then, 100 μl defensin (50 μM) labeled with FITC was added. The plate was incubated for 30 min at room temperature and washed with PBST five times before fluorescence measurement.

For C6/36 cell ELISA, the plates were pretreated with polylysine. Healthy and fresh C6/36 cells were counted and diluted with 0.1 M dicarbonate (pH 9.6) to a final concentration of 1×10^5 cells per well. The plate was processed as described above for JEV structural proteins or defensin-coated ELISA.

Statistical analysis. All experiments were carried out in at least triplicate. Mean values $\pm$ standard deviation (SD) were calculated in Microsoft Excel. Statistical analysis was done with Student's *t* tests and Fisher's method, and *P* values of each independent experiment were calculated by Student's *t* test. The *P* values from independent experiments were then combined by using Fisher's method (33). Values were considered significant when $P < 0.05$. Figures were created in GraphPad Prism 5.0 software.

Data availability. The sequence of *C. pipiens pallens* defensin A used in this study has been released in the NCBI database. The sequence is available in GenBank (accession number MH756645).

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We declare no competing interests.

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